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ORIGINAL ARTICLE

Gas chromatography-mass spectrometry (GC-MS) techniques for metabolic flux analysis of the Bifido shunt pathway*

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Abstract
The presence of the Bifido shunt in *Bifidobacterium* is predicted to lead to the uptake and metabolic conversion of fructose to acetate and lactate. We propose an approach to quantifying the carbon flux through the Bifido shunt by measuring specific ^{13}C-labeled carbohydrate-derived isotopomers by gas chromatography-mass spectrometry (GC-MS). The techniques described may provide an alternative approach for determining the *in vitro* prebiotic potential of dietary oligosaccharides.

Keywords: Bifidobacterium, *Bifido shunt, flux, mass spectrometry, metabolism*

Introduction

During fermentation, nutrient substrates are degraded to simpler compounds that are subsequently used as a source of bioenergetic fuel or as chemical precursors for anabolic growth. These degradation reactions often result in the breaking of carbon–carbon bonds, so that the carbon atoms in nutrients become redistributed during biosynthesis. The extent of this redistribution can be measured by making use of carbon-13 (^{13}C), a non-radioactive isotope that is one mass unit heavier than natural carbon-12 (^{12}C). Carbon-containing compounds, such as sugars, can be isotopically-enriched with ^{13}C, either at selective carbons atoms or universally (U)-labeled (i.e. ^{13}C-enriched at all of the carbon atoms). After uptake the rate of turnover and metabolism of these ^{13}C-labeled sugars into other metabolites can be measured by mass spectrometry.

The concept of probiotics is that an increase in beneficial bacteria in the GI tract may reduce or prevent disease (Gibson 1995). The large intestine is the most heavily colonized region of the digestive tract, and *Bifidobacterium* are generally considered to be among the most beneficial species. A prebiotic is any material that causes a selective increase in the populations of beneficial bacteria; most are oligosaccharides. The enumeration of bacteria as *Bifidobacterium* is difficult, the most direct test is based on demonstration of fructose-6 phosphate phosphoketolase (F6PPK) activity in cellular extracts (Vlkova 2005). This is the key enzyme of the so-called 'Bifido shunt', a unique metabolic pathway that characterizes the genus (Figure 1).

Here we propose a gas chromatography-mass spectroscopy (GC-MS) approach to quantify the carbon flux through the Bifido shunt, by measuring specific carbohydrate-derived metabolites in *Bifidobacterium bifidum*. Metabolites are derivatized suitable for separation by gas chromatography (GC), and the distribution of ^{13}C into various monosaccharides and lipids is determined from the mass spectral (MS) data by isotopomer analysis.

Materials and methods

Growth conditions

Bifidobacterium bifidum NRRL B-1976 was obtained from the Agricultural Research Service Culture

*Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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1. Fructose-6-P + iP – $^{F6PPK}\rightarrow$ 3-P + acetyl-P + $H_2O \rightarrow$ 3 acetate
2. Erythrose-4-P + fructose-6-P – $^{transaldolase}\rightarrow$sedoheptulose-7-P + glyceraldehyde-3-P
3. Sedoheptulose-7-P + glyceraldehyde-3-P – $^{transketolase}\rightarrow$ ribose-5-P + xylulose-5-P
4. 2 xylulose-5-P + iP – $^{xylulose\ phosphoketolase}\rightarrow$ 2 glyceraldehyde-3-P + 2 acetyl-P + $2H_2O$
5. 2 glyceraldehyde-3-P – $^{EMP\ enzymes}\rightarrow$ 2 lactate

Figure 1. Metabolic reactions of the Bifido shunt convert fructose-6-phosphate into lactate and acetate.

Collection at the National Center for Agricultural Utilization Research, Peoria, IL. *B. bifidum* was grown in trypticase (10 gL^{-1})-phytone (5 gL^{-1})-yeast extract (2.5 gL^{-1}) medium containing 0.3% glucose or fructose at 37°C under anaerobic conditions in serum stopper tubes. Bacterial cells were recovered by centrifugation (10,000 $\times g$, 4°C, 10 min) for further analyses.

Derivatization protocols

Cells were suspended in single-phase Bligh–Dyer solvent (chloroform:methanol:water, 1:2:0.8 by volume, converted to two-phase by addition of one volume each of chloroform and water, and centrifuged (2000 $\times g$) to partition (Bligh & Dyer 1959). The upper, aqueous phase was recovered and evaporated to dryness. The sugar-containing residue was hydrolyzed with 2 M trifluoroacetic acid and aldononitrile acetate derivatives were prepared as described previously (Price 2004). The Bligh-Dyer lower phase containing lipophilic metabolites was evaporated and fatty acid pyrrolidide derivates prepared (Andersson & Holman 1975).

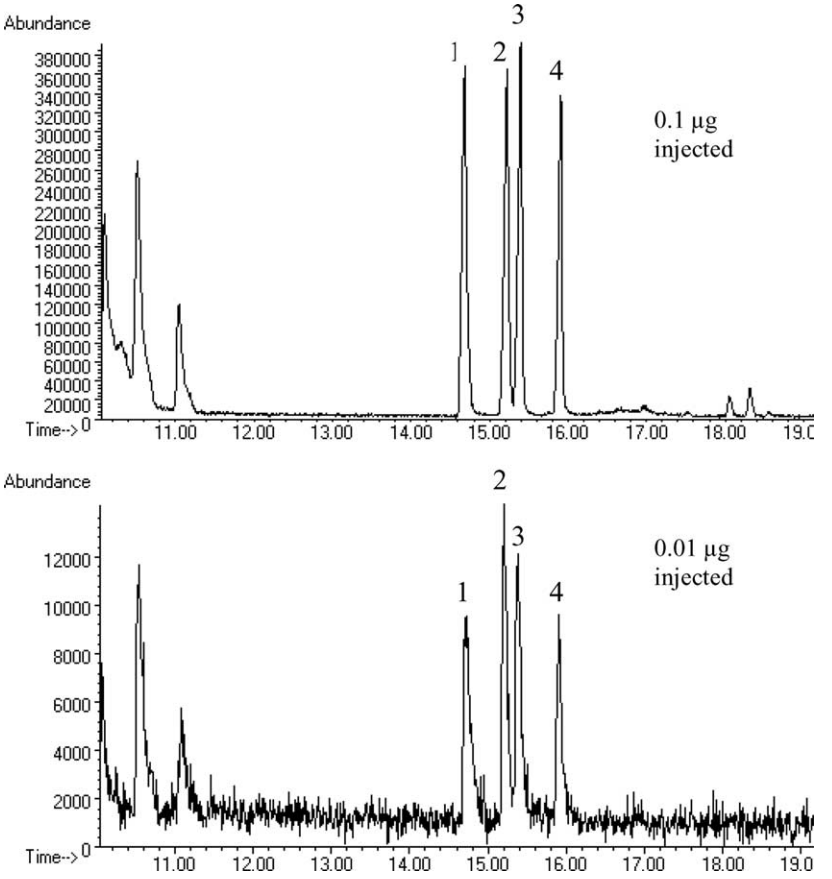

Figure 2. Detection limits for 3-*O*-methyl-glucose (1), mannose (2), glucose (3), and galactose (4) aldononitrile acetates. The injection volumes were 1 µL.

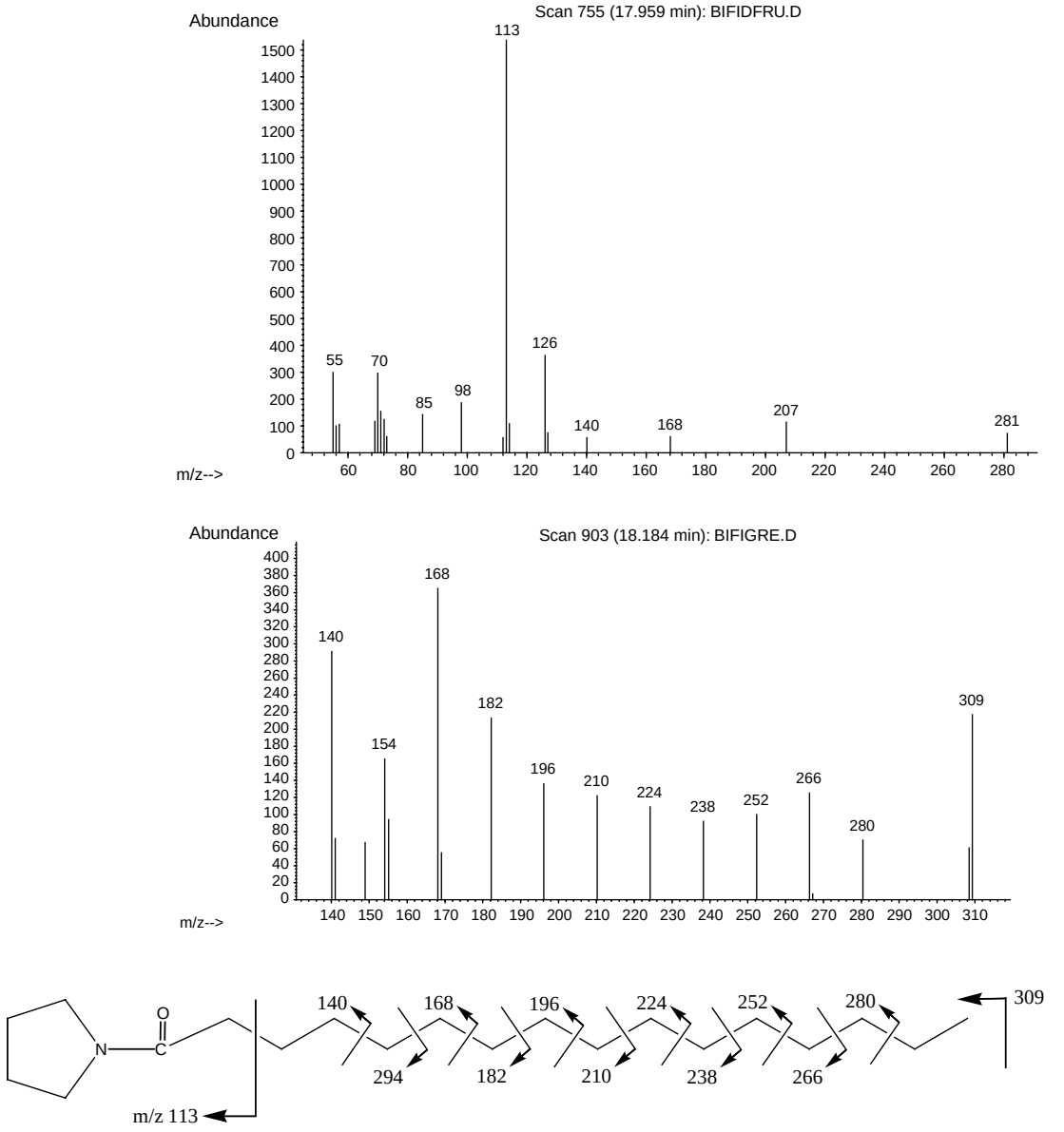

Figure 3. Electron impact mass spectra of palmitoyl pyrrolidide from *Bifidobacterium bifidum.* The characteristic base peak at m/z 113 arises from a McLafferty rearrangement ion.

Mass spectrometry. Ethyl acetate extracts containing sugar aldonitrile acetates or fatty acid pyrrolidides was analyzed by GC-MS on an Agilent 6890N GC system coupled with an Agilent 5973 mass selective detector. An automated injector (Agilent 7683 series) was used to introduce the samples, and ionization was by electron impact (e.i.) mode with positive ion detection. Ion extraction full scale scans were collected over the mass range 50–500 mass units.

Results and discussion

Gas chromatography-mass spectrometry is a well established technique for obtaining positional and quantitative isotopic information (Wittman 2002). The sensitivity is several orders of magnitude better than ^{13}C-NMR and is applicable to a wide range of small metabolites. The requirement is for volatile compounds that undergo well-defined fragmentation pathways on electron impact ionization. Significant carbon–carbon bond cleavage is essential in order to localize $^{13}C/^{12}C$ ratios to specific carbon atoms.

For non-volatile metabolites such as sugars and fatty acids, it is first necessary to form derivatives. Ideally, these should give rise to a single chromatographic peak, without side-reaction impurities. Peracetylation, alditol acetates, trimethylsilylation, aldononitrile acetates and dithioacetatal acetates are

all valuable for GC-MS analysis of sugars, although the latter two are of far greater value for isotopomer determinations. Dithioacetatal acetates, for example, are the only sugar derivatives that fragment across the C1–C2 bond, allowing a direct measure of the $^{13}C/^{12}C$ ratio in carbon 1, the defining anomer carbon (Price 2004). Aldononitrile acetate derivatives can be detected down to 0.01 μg (Figure 2). Peracetylation is not generally applicable to aldose sugars, but has advantage for ketose sugars such as fructose or sorbose (Price 2004).

Fatty acids are generally methylated to methyl esters suitable for analysis by GC (Brondz 2002). These derivatives are often referred to as fatty acid methyl esters (FAMEs). Although well suited for chromatography they give less than ideal fragmentation required for isotopomer determination. By contrast, fatty acid pyrrolidides have several advantages (Andersson & Holman 1975). The amides are readily prepared from salts, esters or free fatty acids, and are directly applicable to analysis of whole cells (Vetter & Walther 1994). The pyrrolidides are well resolved and give rise to characteristic fragmentations in the mass spectrometer. McLafferty rearrangement of the amide head group gives rise to C2–C3 bond cleavage, to generate an intense base peak at m/z 113 (Figure 3). This characteristic ion is readily applicable to ion extraction analysis, or to selective ion monitoring (SIM). Further, ions are generated by carbon–carbon bond cleavage in the fatty acid chain, giving rise to ion series, differing by 14 mass units, i.e. CH_2 (Figure 3). Ion extraction of these masses and M+1 therefore permits isotopic ratios to be measured at each CH_2 within the carbon backbone. This is of advantage for localization of individual isotopic incorporations.

The presence of the Bifido shunt is predicted to lead to the uptake and conversion of [1-^{13}C]fructose to one equivalent of [1-^{13}C]acetate and two equivalents of unlabeled acetate (see Figure 1), the actual fractional incorporation being dependent upon processing of fructose-6-P through the Bifido shunt. This fractionally labeled acetate may be excreted, or incorporated into fatty acid acid metabolism. Hence, extracted phospholipids and fatty acids are expected to become fractionally ^{13}C enriched. In addition, label from ^{13}C fructose-6-P is expected to be re-incorporated into central metabolism via sedoheptulose-7-P and glyceraldehydes-3-P, and subsequently into ribose and xylulose phosphates. Hence the metabolic flux of [1-^{13}C]fructose, [U-^{13}C]glyceraldehyde, and [1-^{13}C]xylose substrates are predicted to be considerably influenced due to the presence of the bifidobacterial F6PPK enzyme.

Prebiotics are compounds which enhance the growth of beneficial, or probiotic, bacteria. Of particular interest are bacteria of the genus *Bifidobacterium*. One key step in evaluating the prebiotic activity of a compound is the measurement of bacterial growth in mixed cultures. Methods in current use include classical plating and colony counting, and fluorescent *in-situ* hydridization (FISH) and microscopic counting of labeled cells (Vulevic et al. 2004). Both of these methods involve certain tedious steps and can be prone to human error. A more accurate method with less likelihood of human error would be of great benefit not only to this research effort, and will provide a more accurate and objective method for evaluating the prebiotic activity of new compounds.

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